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Phenazines affect biofilm formation by *Pseudomonas aeruginosa* in similar ways at various scales

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Abstract

Pseudomonads produce phenazines, a group of small, redox-active compounds with diverse physiological functions. In this study, we compared the phenotypes of *Pseudomonas aeruginosa* strain PA14 and a mutant unable to synthesize phenazines in flow cell and colony biofilms quantitatively. Although phenazine production does not impact the ability of PA14 to attach to surfaces, as has been shown for *Pseudomonas chlororaphis* (Maddula, 2006; Maddula, 2008), it influences swarming motility and the surface-to-volume ratio of mature biofilms. These results indicate that phenazines affect biofilm development across a large range of scales, but in unique ways for different *Pseudomonas* species.

Keywords

Phenazine; Biofilm; Swarming

1. Introduction

Pseudomonas aeruginosa is an important opportunistic human pathogen that causes lung infections in patients with the disease cystic fibrosis (CF) as well as immunocompromised patients (Johansen, 1998). Much attention has been paid to biofilm formation by *P. aeruginosa*, as it is generally accepted that *P. aeruginosa* forms aggregates surrounded by excreted polymeric substances in the CF airway (Govan, 1996). Indeed, biofilm formation has been proposed to be a key to the survival of *P. aeruginosa* in the context of infection (Singh, 2000).

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Traditionally, most researchers have utilized “flow cell” systems to study biofilms in the laboratory (O'Toole, 1999). While the flow cell system allows the fate of individual cells to be monitored over time (Singh, 2002), a major disadvantage to this technique is the impracticality of running many biofilms at once. An alternative approach to studying biofilms that has become more common in recent years is the “colony biofilm” assay (Shapiro, 1984), in which a high cell density suspension of bacteria is applied in a spot onto an agar plate, and the development of the community of cells that grows is observed over time. This assay provides a statistical advantage over the flow cell system in that it is easy to repeat multiple times in parallel. However, unlike flow cells, the colony biofilm assay is not good for following individual cells at early stages of biofilm development. Rather, it is optimal for studying aspects of biofilm development that occur after initial attachment, such as the formation of large, multicellular structures.

Our research focus has been on how phenazines are utilized by *P. aeruginosa* strain PA14. Recently, we discovered that phenazine production dramatically affects *P. aeruginosa* colony morphology, and suggested that this phenotype was due to a complex set of effects, including direct and indirect signaling functions for phenazines (Dietrich, 2008). We wondered whether we would see a similar effect of phenazines on biofilms using a flow cell system. Previous work by Maddula et al. showed that phenazines are involved in flow cell biofilm formation by *P. chlororaphis* strain 30–84 (Maddula, 2006; Maddula, 2008); a lack of phenazine production prevents cell attachment to surfaces and abolishes biofilm formation. *P. aeruginosa* PA14 produces a different set of phenazines than *P. chlororaphis* (Mavrodi, 2001), so whether phenazines would affect its ability to form biofilms similarly was unclear. To probe this, and to determine whether flow cell and colony biofilm phenotypes directly correlate, we undertook the present study.

2. Materials and methods

2.1 Bacterial strains

P. aeruginosa PA14 and its derivatives were used in this study. Strain DKN330, a *ΔphzA1-G1 ΔphzA2-G2* deletion mutant (Dietrich, 2006), is unable to produce any phenazine and is referred to here as *Δphz1/2*. Plasmid pAKN69, containing the mini-Tn7(Gm)$P_{A1/04/03}$::*eyfp* fusion (Lambertsen, 2004), was used to introduce a chromosomally encoded constitutive *e*YFP into PA14 wild type and *Δphz1/2*, resulting in strains DKN372 and DKN373, respectively.

2.2 Cell-surface attachment

Attachment was analyzed using phase contrast imaging on a Leica confocal microscope. Stationary-phase cultures were diluted 1:50 in 10% LB and 0.5 ml of this suspension was pipetted into a sterile chambered system (Lab-Tek, Rochester NY) with a borosilicate cover glass bottom. After 0.5 h or 4 h incubation at 22 °C, unattached cells were discarded by gently replacing the supernatant with fresh medium, and attached cells were counted. Six fields of view for each strain and condition were analyzed and the percent of the surface covered by attached cells was estimated using Adobe Photoshop.

2.3 Motility assays

Swimming, swarming and twitching motilities were determined as previously described (Rashid, 2000).

2.4 Flow cell biofilms

These were grown under constant flow at 22 °C in 1.5 × 4 × 34 mm flow cells. Continuous flow of 10% LB was supplied with a peristaltic pump at a constant rate of 3 ml h^{-1}. An early

stationary phase culture was diluted to an optical density at 500 nm of around 0.1 and 300 µl were inoculated into the flow cell. Strains expressing *eyfp* constitutively were used to visualize the biofilms. Upon inoculation, cells were allowed to attach in the absence of flow for 1.5 h before flow was resumed. Developing biofilms were imaged in 3 dimensions using a Leica confocal microscope. *e*YFP was excited with a 488 nm laser beam kept at constant intensity throughout the experiment, and emission from 510 to 618 nm was collected. Routinely, we observed that the distribution of bacterial cells throughout the colonized surface varied depending on the region of the flow cell, probably due to factors such as flow or accumulation of planktonic cells. To assure reproducibility, all images were acquired from an area set in the middle of the flow cell. Three images of duplicate flow cell lines were recorded and at least two independent experiments were performed.

2.5 Colony biofilms

These were grown on agar containing 1% tryptone as previously described (Dietrich, 2008). Three colonies from independent spottings were documented for 8 days using an Epson scanner.

3. Results and discussion

We tested the ability of *P. aeruginosa* exponential and early stationary phase cells to attach to a borosilicate surface. While cells harvested during exponential growth adhered very poorly, stationary phase cells bound to the surface, establishing stable interactions. In contrast to *P. chlororaphis*, we did not observe significant differences in *P. aeruginosa's* ability to attach as a function of phenazine production (data not shown). Addition of 75 µM pyocyanin or phenazine-1-carboxylate (PCA) to exponential or stationary phase cells did not alter the ability of either the wild type or *Δphz1/2* to adhere to the surface. *P. chlororaphis* and *P. aeruginosa* differ in their phenazine-synthesizing capabilities; while the former produces only two of these compounds (PCA and 2-hydroxyphenazine), the latter synthesizes at least four phenazine varieties, with pyocyanin and PCA being the most abundant (Mavrodi, 2001). Therefore, their distinct synthesizing capabilities may have influenced the way these two pseudomonad species evolved to respond to phenazines.

Previous studies have shown that *P. aeruginosa* attachment to surfaces is mediated by flagella, whereas movement along colonized surfaces is driven by pili, giving rise to cell aggregates that grow to form mature biofilms (O'Toole, 1998). *P. aeruginosa* PA14 is capable of three types of motility: 1) swimming in fluid media, accomplished via reversible rotation of flagella, 2) twitching, a surface-associated movement mediated by type IV pili and 3) swarming, which also occurs on solid surfaces and is dependent on flagella. We found that swimming and twitching motilities of the *Δphz1/2* mutant were indistinguishable from the wild-type strain (data not shown). However, swarming motility was significantly higher in *Δphz1/2* (Fig. 1). After 40 h incubation, the *Δphz1/2* mutant covered on average 84% more surface than the wild type. Addition of 100 µM PCA to the swarming agar had no effect on the wild type, but it significantly decreased the motility of the *Δphz1/2* mutant. Addition of 100 µM pyocyanin had no inhibitory effect on swarming of either the wild type or *Δphz1/2*. Altered swarming motility often correlates with differences in rhamnolipid or other surfactant production (Deziel, 2003). Using a drop-collapse assay we were unable to observe significant difference in surfactant production between the wild-type and *Δphz1/2* strains.

To measure the impact of phenazines on biofilm formation directly, we first used a flow cell system to grow wild-type and *Δphz1/2* biofilms. In this system, the wild type formed heterogeneous biofilms that after 4 days developed large and abundant microcolonies (Fig. 2A). Biofilms of *Δphz1/2* were flatter and consisted of fewer and smaller aggregates scattered throughout the field of view (Fig. 2B). The morphology of 4-day-old biofilms was analyzed

using COMSTAT (Heydorn, 2000). A fixed threshold value and connected volume filtration were used for all image stacks. Table 1 summarizes the values calculated for mean biofilm thickness, substratum coverage, number of microcolonies at the substratum, surface-to-volume ratio and maximum biofilm thickness for three independent experiments. While the wild type showed a higher maximum biofilm thickness than *Δphz1/2*, the phenazine-deficient mutant showed a higher surface-to-volume ratio. When *Δphz1/2* biofilms were grown in the presence of 25 μM pyocyanin (Fig. 2C), larger microcolonies and thicker biofilms resulted. PCA was not tested under these conditions. The mean thickness, the number of microcolonies and the total biomass volume all increased, reaching numbers similar to or even higher than those observed for the wild type (Table 1). These results indicate that pyocyanin actively shapes the architecture of *P. aeruginosa* flow cell biofilms.

To determine whether the morphological trends we observed in flow cells might translate to a larger scale, we grew colony biofilms of the wild type and *Δphz1/2*. As we reported previously (Dietrich, 2008), wild type PA14 formed smooth colonies that developed concentric ridges only after prolonged incubation (4 days; Fig. 3A), while *Δphz1/2* formed wrinkled colonies that grew vertically within 2 days. Surface coverage by *Δphz1/2* colonies was increased by up to 75% compared to wild-type colonies after 7 days (Fig. 3B). These features are consistent with an increased surface-to-volume ratio in the absence of phenazines, mirroring our findings for flow cell biofilms. We tested PCA and pyocyanin for their specific effects on surface coverage and rugosity by adding 0.2 M of either compound to the agar. Both phenazines significantly decreased rugosity and surface coverage of *Δphz1/2* colonies, while they did not noticeably affect the structure of wild-type colonies (Fig. 3 A, B). Interestingly, PCA prevented colony spreading and the formation of wrinkles in *Δphz1/2* colonies more efficiently than pyocyanin. A titration of phenazines showed that 0.1M PCA was sufficient to decrease surface coverage of *Δphz1/2* colonies to wild-type levels (Fig. 3C), and higher concentrations of PCA (up to 0.4M) had no additional effects. In contrast, *Δphz1/2* colonies still showed 50% increased surface coverage compared to wild-type colonies in the presence of 0.1 M pyocyanin. At pyocyanin concentrations of 0.2M or higher, *Δphz1/2* covered 35% more of the agar surface than wild-type colonies. Therefore, the nature of the phenazine, in addition to the total amount of phenazine, is an important parameter influencing colony structure.

When we began this study, it was not clear at which stage phenazines would manifest their effect for *P. aeruginosa* PA14. Prior work by Maddula and colleagues (Maddula, 2006; Maddula, 2008) showed that phenazine production in general, and the ratio of the specific phenazines produced, dramatically affected both the attachment and thickness of mature biofilms formed by *P. chlororaphis* 30–84 in flow cells. Thus, we were surprised that phenazine production did not affect the ability of PA14 to adhere to surfaces. This suggested that a later step in biofilm formation in PA14 was responsive to phenazines. Consistent with this idea, we found that swarming was significantly different between the wild type and phenazine mutant (*Δphz1/2*) and that this difference in swarming motility correlated with differences in biofilm morphological development.

In flow cells, the *Δphz1/2* mutant grew thinner biofilms in which a greater proportion of the biomass was exposed to the nutrient flow, as indicated by the significantly higher surface-to-volume ratio calculated for this strain (Table 1). The average size and number of microcolonies formed in wild-type biofilms was significantly higher. However, the calculated roughness coefficient, a measure of the biofilm topology, was lower than that of the *Δphz1/2* mutant. The formation of smaller aggregates, not considered in the colony count but seen in Fig. 2B, might account for the increase in roughness and surface-to-volume ratio of the *Δphz1/2* communities. We observed similar trends for the colony biofilms: *Δphz1/2* developed highly rugose colonies and an increased surface-to-volume ratio over time. In contrast, the wild type formed smoother colonies that showed less expansion and wrinkling over the course of the experiment (Fig. 3).

The addition of phenazines to the medium on which the *Δphz1/2* mutant was grown restored the general attributes of wild type biofilms regardless of whether they formed in a flow cell or on an agar plate (Fig. 2 and 3 and Table 1). The mechanism(s) by which phenazines influence biofilm architecture and swarming are unknown. However, the observation that rhamnolipid production was not significantly altered in the *Δphz1/2* mutant suggests that phenazines might regulate flagellar function.

Despite the practical advantage colony biofilms provide for studying certain aspects of biofilm development, they are sometimes dismissed as not being "true" biofilms because they do not develop under a regime of fluid flow. This study challenges that assumption for *P. aeruginosa* biofilms, as quantitatively similar features can be observed at later stages of development regardless of how the biofilms are grown.

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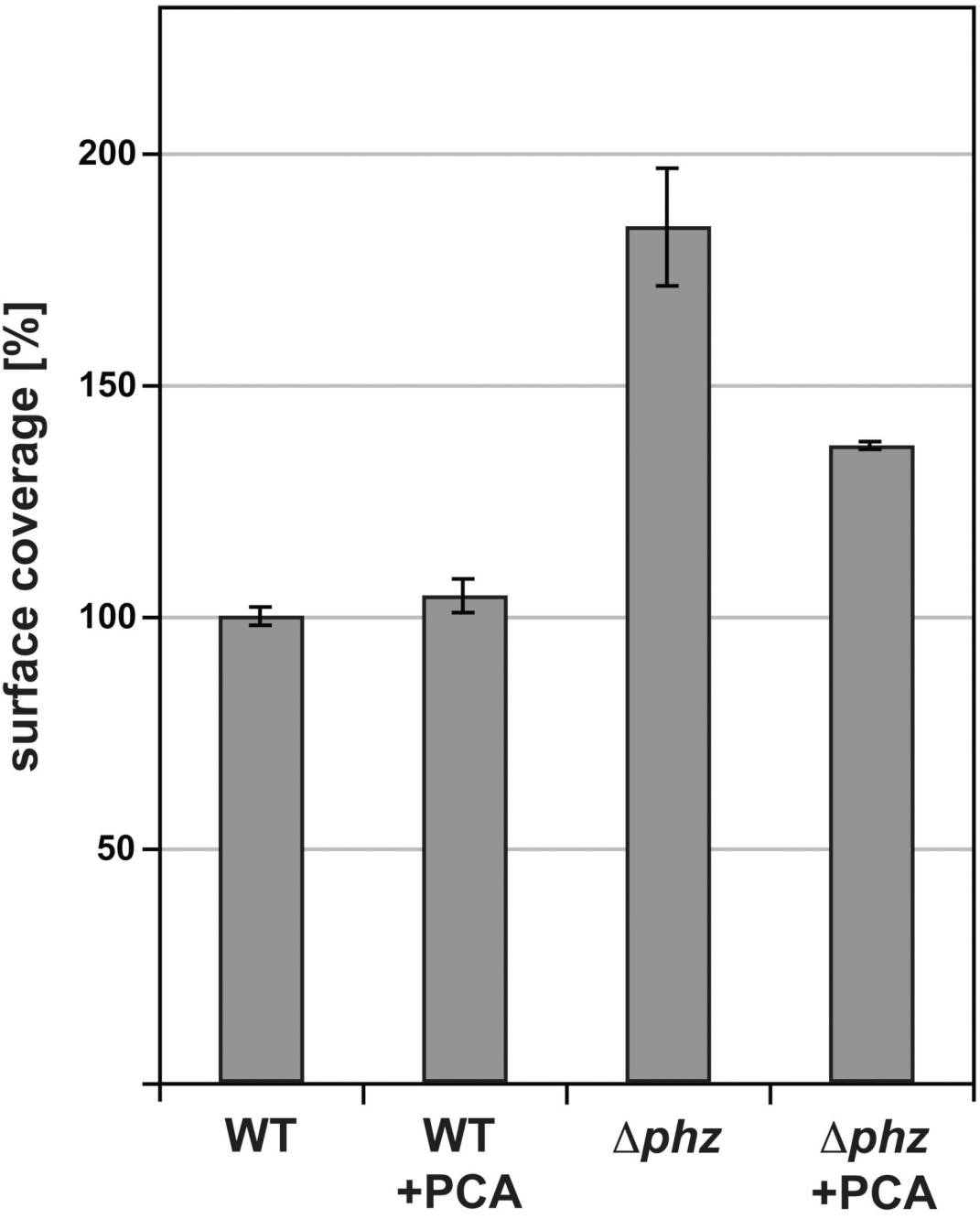

Fig. 1. Δ*phz1/2* has increased swarming motility

Quantification of swarming motility. Images of three swarming plates for each strain or condition were captured, exported to Adobe Photoshop and the agar surface covered by the swarms was quantified using Analysis Tools.

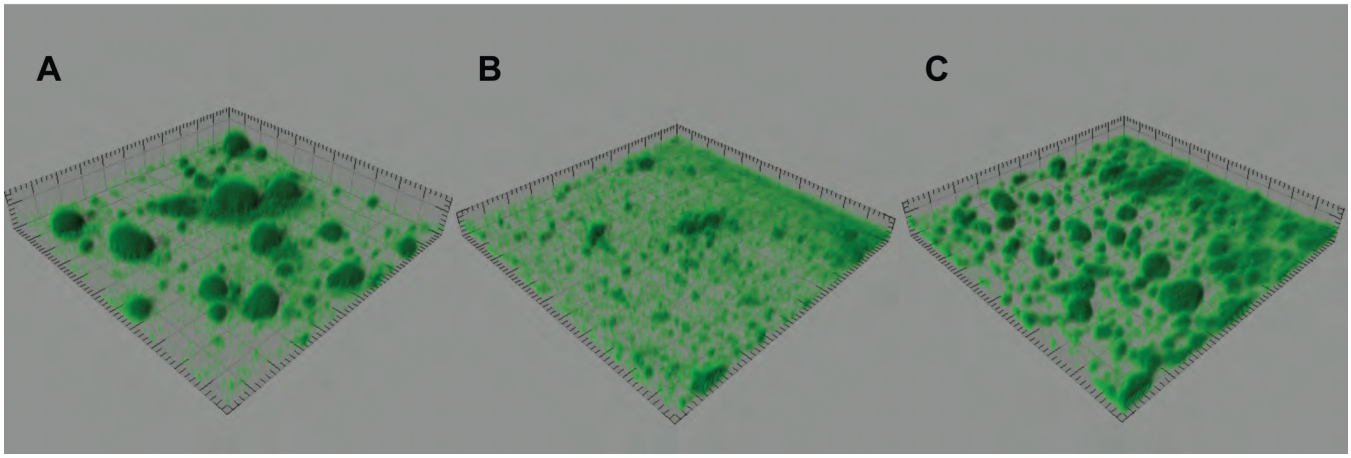

Fig. 2. Phenazines support development of structured biofilms in a continuous flow cell-system

Representative images of 4-day-old biofilms of the wild-type (A) and *phz1/2* mutant without (B) and with addition of 25 μM pyocyanin (C) to the nutrient flow. Images of similar positions in the flow cell were taken in triplicate. Three independent experiments were performed with similar results.

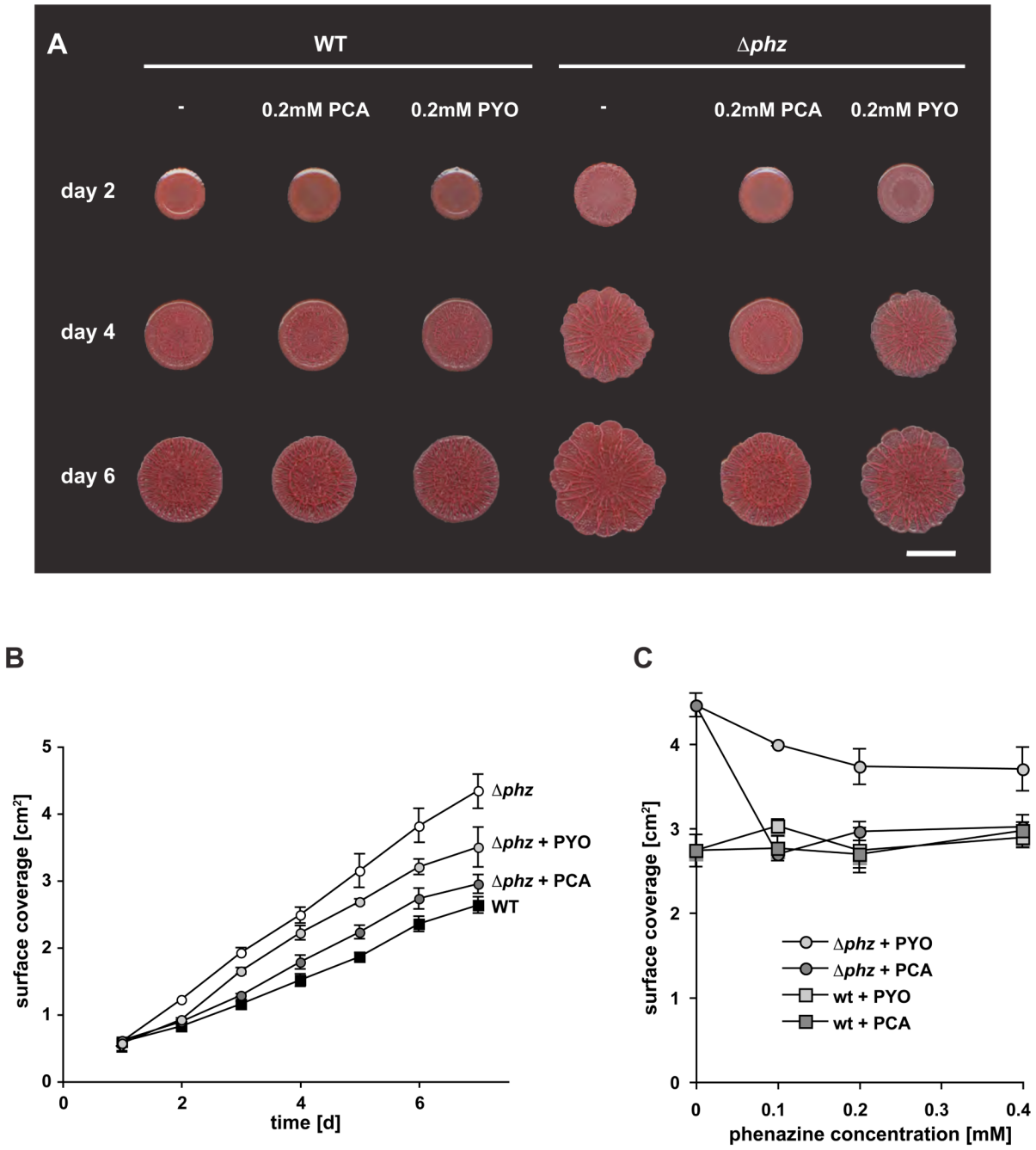

Fig. 3. Phenazines modulate colony biofilm morphology

(A, B) *P. aeruginosa* PA14 wild-type and Δ*phz1/2* cultures were spotted onto 1% agar plates (containing 1% tryptone, 40 μg/ml Congo Red and 20 μg/ml Coomassie Blue) and incubated at 20°C for 7 days in the presence of 0.1 mM PCA, 0.1 mM or in the absence of supplements. Each day, colonies from three independent experiments were scanned and their surface coverage was determined. Representative images of colonies at days 2, 4 and 6 are shown (A; scale bar is 1 cm). The data in (B) show the average surface coverage and standard deviation for the three independent experiments. (C) Phenazine titrations. Colonies were grown as above, supplemented with 0, 0.1, 0.2, 0.3 or 0.4 mM PCA or pyocyanin. Surface coverage of colonies

from three independent experiments was determined after incubation at 20°C for 7 days. Bars indicate the standard deviation.

Table 1

Quantitative analysis of 4-day-old biofilms formed by the wild-type and a phenazine-defective mutant.

	Wild-type	***Δphz1/2***	***Δphz1/2*** **PYO** [a]
Total biomass ($\mu m^3/\mu m^2$)	2.2 ± 0.49	0.41 ± 0.04	4.3 ± 1
% Coverage at substratum	8 ± 1	2.7 ± 0.6	15.2 ± 2.46
Maximum thickness	54.7 ± 6.4	40 ± 2	57.5 ± 1.9
Average thickness	2.4 ± 0.59	0.47 ± 0.03	5.6 ± 1.6
Number of microcolonies [b]	9.3 ± 3	2	8 ± 1.5
Average size of colonies at substratum (μm^2)	46.4 ± 13	12.1	102.6 ± 31
Average colony volume (μm^3)	1551 ± 498	251.9	8170
Roughness coefficient	1.75	1.9	1.3
Surface to volume ratio ($\mu m^2/\mu m^3$)	3.5 ± 0.23	10.5 ± 3.6	6.6 ± 0.6

Analysis was conducted using COMSTAT. Images from similar positions in the flow cell were acquired for all conditions in triplicate.

[a] 25 μM pyocyanin (PYO) was added to the medium from the beginning of the experiment.

[b] Minimum microcolony size at the substratum was set at 10 μm^2.